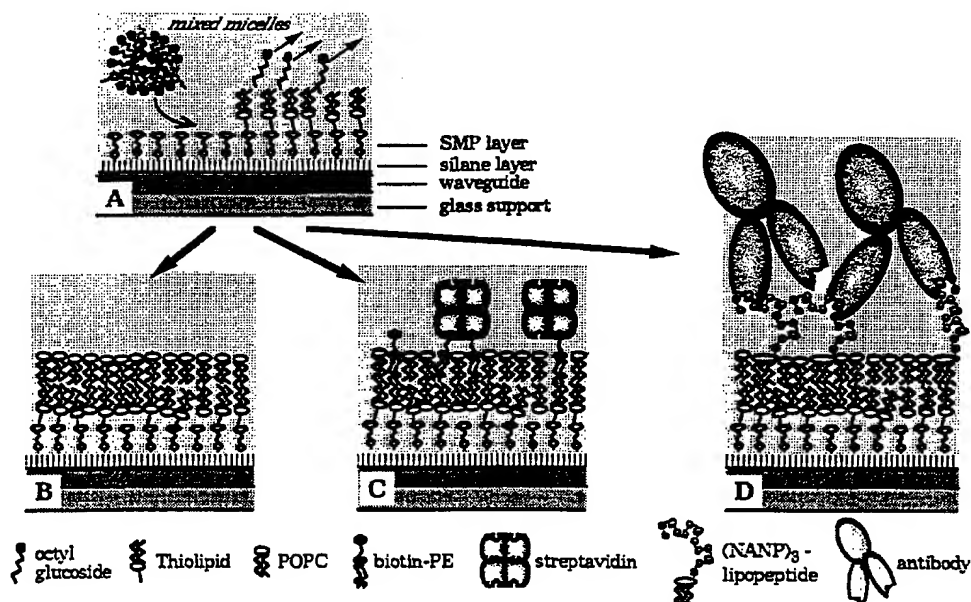




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(54) Title: COVALENTLY IMMOBILIZED PHOSPHOLIPID BILAYERS ON SOLID SURFACES



(57) Abstract

A solid coated device having covalently attached a coating which comprises a first layer containing first functional groups, to which first layer functional groups is covalently attached a second, linking layer carrying second functional groups, to which second, linking layer is covalently attached a third, proximal phospholipid layer, which third, proximal phospholipid layer, to which third, proximal phospholipid layer is not-covalently attached a fourth, distal lipid layer, so that the proximal and the distal lipid layers together form a bilayer, which lipid bilayer, into which lipid bilayer are optionally inserted receptor molecules, method of the preparation thereof, and uses thereof as biosensors or implantation devices.

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COVALENTLY IMMOBILIZED PHOSPHOLIPID BILAYERS ON SOLID SURFACES

Field of the Invention

5

The invention concerns devices carrying on the surface covalently immobilized lipid bilayers which bilayers optionally contain biological receptor molecules, processes for their preparation, and their use as biosensors or
10 implantation devices.

Background Art

The common structural element of cell membranes is a double
15 layer (herein "bilayer") of lipid molecules held in place by intermolecular forces. A lipid is generally defined as a molecule carrying at one end a hydrophobic hydrocarbon chain, and at the other end a hydrophilic polar group. Membranes separate compartments, each membrane being associated with an
20 inside and an outside. Many biologically important signal transduction processes occur at the level of cell membranes. Specialized membrane receptors selectively detect and bind ligand and thereby filter these extracellular signals, pass them on across membranes, amplify and integrate them.

25

Supported lipid bilayers with reconstituted membrane-bound receptor molecules are potentially useful as receptive layers in biosensors. A biosensor is a device which converts biological activity into a quantifiable signal. In the
30 receptor part of a biosensor the (bio)chemical information is transformed into a form of energy which is measured by the transducer. The transducer part is a device capable of transmitting this energy. It transduces the chemical information from the sample to an analytical signal.

Transducer systems include electrochemical devices, piezo-electric crystals, surface acoustic devices, thermistors and optical devices.

5 The clinical application of sensing devices or artificial biomaterials which contact body fluids is of major importance in modern medicine. However, it is well known that proteins and cells adsorb on the surface of artificial materials when they come in contact with blood or other body fluids. Adverse
10 reactions between foreign or prosthetic surfaces and blood components are the predominant factors restricting the use of certain biomaterials. A typical example of this is the inability of biosensors to operate effectively for any length of time in blood.

15 Several attempts have been made to enhance the biocompatibility of implant materials by coating them with layers of naturally occurring or synthetic lipids. For example, Kono et al. (1989) reported suppressed platelet
20 adhesion to polyamide microcapsules coated with neutrally charged lipid bilayer membranes compared to platelet adhesion to bare polyamide surfaces. Chapman (1993) reported a suppressed thrombogenicity of lipid bilayer coated plastic or metal surfaces. It is therefore believed that coating
25 biosensor surfaces with lipid bilayers will distinctly enhance their potential applicability in body fluids, either for in vitro assays or as implantable devices.

At present, there are three techniques published to create
30 supported lipid bilayers:

(i) The Langmuir-Blodgett technique (LB-technique) is suited to create supported lipid bilayers in a layer by layer manner. For reviews see McConnel et al., 1986, and Roberts,
35 1992.

(ii) Several partially modified self-assembly (SA) techniques of lipid vesicles, applicable to hydrophilic surfaces, which result in a supported lipid bilayer by a surface-induced fusion of the lipid vesicles have been described by Bayerl et al., 1990, Kiefer et al., 1991, Johnson et al., 1991, and Contino et al., 1994. A modified procedure was applied to solid surfaces which are totally or partially covered by a molecular layer of physisorbed fatty acids or phospholipids transferred by LB-techniques to hydrophilic surfaces (Kalb et al. 1992) or by SA of thioalkanes or thiolipids (Lang et al. 1994) to gold or silver surfaces resulting in a hydrophilic surface of the supporting material. In a second step the first layer is completed and simultaneously a second lipid layer is formed (Lang et al. 1994).

(iii) The formation of supported lipid layers on a solid support, which is totally or partially covered by a first hydrophobic molecular layer (see ii) can be also formed by SA of mixed lipid-detergent micelles by a simple dilution technique below the critical micellar concentration (Lang et al. 1994, and Terrettaz et al. 1993).

Supported lipid bilayers as membrane models for studying ligand-receptor interactions occurring at cell membrane surfaces have been introduced by McConnell (1986). Lipid bilayers on solid supports represent geometrically well defined systems: the hydrophilic polar groups of the lower (proximal) lipid monolayer contact the support, whereas the polar groups of the upper (distal) monolayer face the surrounding aqueous phase. Hydrophobic forces hold together the two lipid leaflets.

The incorporation of naturally occurring receptor molecules in a functionally active state in supported lipid layers formed the basis of pioneering biosensors, which made use of transmembrane proteins and glycolipids as receptor molecules.

Procedures for the covalent attachment of lipid bilayers have been realized by Lang et al., WO 93/21528, in the case of gold-surfaces, exploiting the strong interactions (chemisorption) between sulfur-bearing molecules and a zero-valent gold surface. However, many device surfaces are not composed of zero-valent metals. For such surfaces other methods for the covalent attachment of lipid bilayers have to be followed.

Two studies dealt so far with the covalent attachment of lipid bilayers on surfaces composed of materials other than zero-valent metals: Uzgiris (1987) published UV immobilized phospholipids bilayers, where dinitrophenyl phosphatidylethanolamine containing monolayers, which were deposited by LB-technique onto carbon shadowed nitrocellulose, became covalently linked to the support upon irradiation at 350 nm (Fig. 1a in Uzgiris; 1987). However, the experimental evidence for a covalent linkage of the lipid layer is very poor. Uzgiris admits that a second layer, deposited onto the first, covalently bound layer, could not be removed by diluted detergent solutions or by organic solvents. In Uzgiris' method the photoactivatable headgroups of the reactive lipids in the lower leaflet are in direct contact with the support. The direct attachment of the first proximal monolayer to the support results in a very inflexible and rigid membrane which lacks the space and water required for the proper folding of the extra-membraneous parts of membrane proteins. In addition the described photoimmobilization procedure (radical formation) has not been applied to hydroxylated surfaces.

Another concept for the covalent attachment of lipid bilayers was presented by Erdelen et al. (1992) on the 4th European Conference on Organized Thin Films. This approach comprises the modification of glass, silica or quartz with a monolayer of isothiocyanate bearing silanes. Double chain lipids with a hydrophilic polyethylenoxide spacer and terminal amino groups

were then covalently linked to the thiol activated support via thiourea formation. Afterwards a second lipid layer is transferred via LB-technique. Erdelen et al. apply a synthetic double-chain lipid in the polar head. No phospholipids were employed but only lipids with terminal amino groups. However, phospholipids are the most abundant lipid species in cell membranes.

It has been frequently observed that the reconstitution and biological activity of membrane anchored- and transmembrane proteins are optimal when they are embedded in a fluid phospholipid bilayer membrane (Gennis, 1989). Phospholipids are therefore indispensable building-blocks when designing supported lipid bilayers which closely mimic cell membranes with regard to their function and structure.

Thompson et al., USP 4,824,529, disclose a protected lipid membrane-based device comprising a porous membrane-protective layer to which is physically attached a lipid membrane. Harden, USP 4,490,216, describes a device sensitive to polarity comprising a solid electrically conductive layer to which is covalently attached an alkylsilane as a first layer and to which is non-covalently attached a lipid to form a mixed bilayer membrane. Ulrich et al., USP 4,637,861, disclose a lipid monolayer covalently bound to a support. Osman et al., WO 90/02327, disclose improvements in sensitivity and selectivity of ion channel membrane biosensors. Gitler et al., EP 441 120 disclose biosensors with a lipid bilayer on a recording electrode. King et al., WO 92/17788, provide electrode membrane combinations for use in biosensors. Cornell et al., WO 89/01159, describe amphiphilic bilayer membranes with a plurality of ion channels incorporated claimed to be useful as biosensors.

None of the prior art discloses the novel and improved devices envisioned in the present invention and the uses thereof.

5 Object of the Invention

It is an object of the present invention to provide novel and improved solid devices coated with a phospholipid bilayer of which the proximal lipid layer is stably attached to the
10 basic material in a distance from the surface of the device to allow the presence of an aqueous layer between the bilayer and the surface of the device and to allow for easy incorporation of large receptor molecules.

15 It is a further object to incorporate bioreceptors into such bilayer in a mode that naturally occurring conditions of biological membranes are mimicked and substrate/receptor binding can be measured by electrical or optical methods.

20 It is a further object to provide simple and reproducible methods for the production of such coated devices and to provide uses for the coated devices.

Detailed Description

25

The present invention concerns a solid device carrying a coating which coating comprises

- (a) a first covalently attached layer containing first functional groups,
- 30 (b) to which first layer functional groups is covalently attached a second, linking layer carrying second functional groups,
- (c) to which second, linking layer is covalently attached a third, proximal phospholipid layer,

(d) to which third, proximal phospholipid layer is non-covalently attached a fourth, distal lipid layer, so that the proximal and the distal lipid layers together form a lipid bilayer,

5 (e) into which lipid bilayer are optionally inserted receptor molecules.

The invention is clearly illustrated by the accompanying Figures of which a short description follows.

10

Short description of the Figures

Fig. 1: Schematic cross-sections of different waveguide devices useful as biosensors.

15 (A) Binding of thiolipids from mixed micelles to a maleimide-modified waveguide surface to form an imperfect third, proximal lipid layer.

(B) Biosensor with fourth, distal lipid layer on the third, proximal lipid layer, self-assembled from vesicles or mixed
20 micelles, forming a lipid bilayer linked to the waveguide surface via linker molecules. Lipids of the fourth, distal lipid layer also fill up the imperfect third, proximal lipid layer.

(C) Self-assembled lipid bilayer containing a fraction of
25 biotinylated lipids to which streptavidin is bound.

(D) Self-assembled lipid bilayer containing a fraction of (NANP)₃-lipopeptide as lipid-anchored peptide antigen to which specifically and nonspecificly a monoclonal anti-(NANP)_n antibody is bound.

30 Fig. 2: Representation of an example of the four layers of the coat of a coated device. X represents a group $\text{CH}_2\text{N}(\text{CH}_3)_3^+$, a chemically reactive group, a receptor molecule, e.g. biotin, a carbohydrate, or a polymer.

Fig. 3: The graph shows the change of the apparent thicknesses during formation of the lipid layers versus time and finally the binding of a streptavidin layer to the lipid bilayer on a maleimide-modified waveguide as measured with the integrated Optics Scanner from ASI AG .

(A) The proximal lipid layer consists of the thiolipid DOPSH. Fifteen to sixty minutes after its attachment a steady state is reached. The actual average thickness of the thiolipid layer bound to the surface is evaluated as the difference between the signal of the buffer before adding DOPSH micelles and the measured stable signal of the thiolipid layer after the second washing step (end of period A). This value was arbitrarily defined as 0 Å on the thickness axis. Accordingly, the measurement starts at negative values. The two polarizations of the laser light (TE- and TM-modes) yield different thickness values for the isotropically calculated thiolipid layer. This can only be explained by the assumption that the thiolipid binding changes the overall anisotropy of the system.

20

(B) Self-assembly of the fourth, distal lipid layer from POPC vesicles doped with 2 mol% biotin-DPPE.

(C) After rinsing with buffer, streptavidin solution is added, resulting in an increase of the apparant thickness.

Fig. 4: The graph shows the change of the apparent thicknesses versus time of the fourth, proximal lipid layer containing (NANP)₃-lipopeptide during formation of said layer and after addition of anti-(NANP)_n antibody on a waveguide surface as measured with the integrated Optics Scanner from ASI AG.

30

(A) After one washing step with octyl glucoside (7-15 min), a second lipid layer is formed by applying a 50 mM octyl glucoside solution thereof.

5 (B) The buffer is exchanged for the antibody buffer, causing a change in the index of refraction of the medium. Addition of a solution of antibody resulting in a continuous binding reaction.

10 (C) Part of this antibody is displaced upon addition of free antigen (NANP)₆ resulting in a decrease of the thickness of the added layer.

The invention is further described in the following in more
15 detail, providing preferred embodiments, a number of examples and results.

A solid coated device according to the present invention is amenable for electrical or optical signal detection and
20 propagation. It can be used with membrane incorporated receptor molecules as a biosensor, which can selectively bind drugs, hormones, proteins, viruses etc.

The biosensor devices amenable for electrical signal
25 detection are such wherein the lipid bilayer with incorporated receptor molecules is electrically coupled to a transducer such that changes in electrical resistance and capacitance of an electrode upon which the bilayer is mounted can be monitored.

30 The biosensor devices amenable for optical signal detection are such wherein the lipid bilayer with incorporated receptor molecules is in intimate contact with a transducer such that the binding of ligand molecules to the receptors can be opti-

cally monitored. This is conveniently performed by measuring the changes in the effective refractive indices of guided modes using waveguiding techniques.

- 5 The devices of the present invention are further permanently or temporary implantable devices for humans and animals, such as, pace makers, artificial metal or polymer joins, catheters, and the like.
- 10 The basic uncoated material of such devices consists, depending on its prospected use, of metal, e.g. titanium, metal oxides, alloy, glas, ceramic or a polymer material. The surface of the basic uncoated device, eventually only in form of a film, is composed for example and without limitation, of
- 15 glass, diamond or diamond-like materials, silicium, silicium dioxide (SiO_2), silicon nitride (e. g. Si_3N_4), tantalumoxide (Ta_2O_5), titanium dioxide (TiO_2), titanium nitride, titanium carbide, platinum, tungsten, aluminum, or indium/tin oxide and carries on the surface functional groups, such as
- 20 carboxyl, amino, thiol or in particular hydroxyl groups, to which the coating can be covalently attached. Such basic materials carrying such functional groups are known in the art or can be produced according to conventional methods. For example, a silicon nitride film with varying amounts of NH-
- 25 groups can be produced from SiH_4 and NH_3 by plasma discharge deposition according to Gmelin (1995) or Efimov et al. (1992).

- Uncoated devices for biosensor use are commercially availabel
- 30 sensor chips, e.g, planar optical waveguides having a $\text{TiO}_2\text{:SiO}_2$ 2:1 surface, obtainable e.g. from ASI AG, Zürich, Switzerland, or optical fibers, obtainable e.g. from ATOF Ag, Luzern, Switzerland.

The devices of the present invention are further permanently or temporary implantable devices for humans and animals, such as pace makers, artificial metal or polymer joins, catheters, and the like. Uncoated devices for implantation use are
5 suited for implantation into a human or animal, and consist of a metal, such as a biocompatible metal, e.g. titanium, aluminum, platinum, or a platinum alloy, a ceramic material, or a biocompatible organic polymer, e.g. polyurethane, poly(methyl methacrylate), polyethyleneterephthalate (PET),
10 polytetra-fluoroethylene (PTFE,). They are commercially available, e.g. from Sulzer AG, Switzerland.

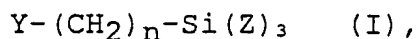
The composition and formation of the different layers of the coating are described in the following steps.

15

Step 1: Formation of the first layer containing first functional groups

The first layer is covalently attached to the functional groups of the basic device in a manner known per se. It is
20 preferably created by reacting the surface of the basic device with a reactive organosilane carrying a first functional group on the other end. Many functionalized organosilan compounds are available. Silylation of various types of surfaces with alkoxysilanes carrying functional
25 groups has been disclosed e.g. by Kallury et al. (1989). Preferred silane coupling agents for creating a first layer containing first functional groups are for example of the formula

30



wherein Y is an optionally protected functional group, such as amino, protected amino, hydroxy, protected hydroxy, mercapto, protected mercapto, carboxyl or protected carboxyl,

Z is lower alkoxy having from 1 to 6 carbon atoms, such as in particular methoxy, or ethoxy, propoxy, butoxy, pentoxy or hexoxy, or halogen such as Cl, Br or J, and n is an integer from 1 to 8, in particular 3. Preferably such agents are
5 amino-lower alkyl-tri-lower alkoxy silanes, wherein lower alkyl has from 1 to 8 carbon atoms, and is e.g. methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl or octyl, and lower alkoxy has from 1 to 3 carbon atoms, and is e.g. methoxy, ethox or propoxy. Particular preferred silanization compounds
10 are 3-aminopropyltriethoxysilane and 3-mercaptopropyltrimethoxysilane, resulting in an "amino device surface" or a "thiol device surface", respectively.

Activation of the device surface, leading to an increased
15 number of surface exposed hydroxyl functions, is preferably performed prior to silane coupling. Siloxane films on the device surface are preferably formed by reacting the device in an organic solvent containing the silane coupling agent for several hours at elevated temperatures. Alternatively,
20 silane coupling is performed by exposing the device surface to the silane in the vapor-phase.

If necessary the protecting groups are removed before the next step.

25

Step 2: Formation of second, linking layer containing second functional groups

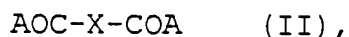
To the functional groups of the first layer is covalently attached the second, linking layer by a conventional method.
30 The linking layer containing second functional groups is created by reacting the first functional groups, with a homobifunctional- or heterobifunctional crosslinking agent. Homobifunctional crosslinking agents carry at both ends of the molecules identical functional groups suitable for

reacting at first with the functional groups of the first layer and subsequently with the headgroup functions of the phospholipids of the third layer.

- 5 Preferred homobifunctional crosslinking agents are for example

activated alpha,omega-dicarboxylic acid esters or anhydrides of the formula

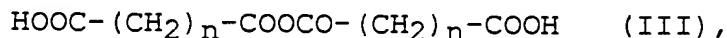
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wherein A is an activating group, such as preferably N-succinimidylloxy, or alkyl- or arylcarbonyloxy, halogen, e.g. 15 chloro, bromo or iodo, alkan- or arylsulfonyloxy, e.g. methan- or benzolsulfonyloxy, or the two A together represent oxygen to form an inner anhydride, X is a group $(\text{CH}_2)_n$, wherein n is an integer from 1 to about 12, preferably 2 to 6, in particular 6, or X is a group $(\text{CH}_2)_m\text{COO}-(\text{CH}_2)_m\text{-OCO}-(\text{CH}_2)_m$, 20 wherein m is an integer from 2 to 4, preferably 2,

dicarboxylic acid anhydrides of the formula

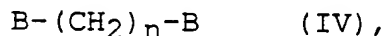
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wherein n is an integer from 1 to about 12, preferably 2 to 6, in particular 2, or

bismaleimidoalkanes of the formula

30



wherein B is the 1-maleimido rest and n is an integer from 2 to 12, preferably 4 to 8, in particular 6.

35

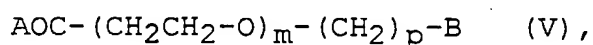
Accordingly, said second functional groups of the linking layer are in particular a N-succinimidyloxy ester group, or another activated carboxylic acid ester group, a carboxylic acid group or a maleimido group.

5

Heterobifunctional crosslinking agents carry at each end of the molecule different functional groups: one of them is selectively reactive with the functional groups of the first layer whereas the other functional group is selectively
10 reactive with the headgroup function of the phospholipids of the third, proximal phospholipid layer.

Preferred heterobifunctional crosslinking agents carry for example at one end a succinimidyloxy ester function, suitable
15 for reacting with primary amines, and at the other end a 1-maleimido group, the double bond of which being suitable for addition reactions with thiols. Preferred heterobifunctional crosslinking agents are of the formula

20



wherein A is an activating group, such as preferably N-succinimidyloxy, or alkyl- or arylcarbonyloxy, halogen, e.g. chloro, bromo or iodo, alkan- or arylsulfonyloxy, e.g. methan-
25 or benzolsulfonyloxy, B is the 1-maleimido rest, m is an integer from 0 to 12, preferably 0 to 6, and p is an integer from 1 to about 6, preferably 2 to 4, in particular 2, whereby m is selected depending on the degree of the desired hydrophilicity, and m and p together are selected on the
30 distance desired between the surface of the uncoated device and the third, proximal lipid layer.

The linker group according to formula (V), wherein m is above
> 0 is of hydrophilic nature.

35

The first layer together with the second, linking layer separate the third, proximal phospholipid layer from the surface of the uncoated device depending on the length of the carbon chains expressed by the integers n, m and p. Said
5 integers can be adapted to the size and structure of the receptor protein so that the distance of the proximal phospholipid layer to the surface of the device leaves sufficient space for the receptor protein.

10 Reaction of one of the functional groups of the crosslinking agents with the functional groups of the first layer is achieved in a conventional manner, depending on the functional group of the first layer and the crosslinking agent. The application of heterobifunctional crosslinking
15 agents for tethering proteins to surfaces has been for instance described by Hong et al (1994).

Activated esters and anhydrides of the formulas II to V react easily with a hydroxy or amino group in an organic solvent or
20 organic solvent/buffer mix, if need be in the presence of a condensing catalyst agent. After the reaction with the functional groups of the first layer, the excess of crosslinking agent is removed by washing procedures.

25 In case the functional group of the first layer is a carboxyl group, it at first, in case it is protected, should be deprotected and transformed into an activated carboxyl group, such as a group -COA described above. This activated group may react with a crosslinker agent carrying instead of a
30 group -COA a hydroxy or amino group.

Preferably the amino group of the first layer is reacted with a heterobifunctional crosslinker of the formula IV, wherein A is succinimidyl, to give a linking layer which is
35 covalently bound by an amide bond to the amino group of the

first layer and which has a maleimido functional group at the outside.

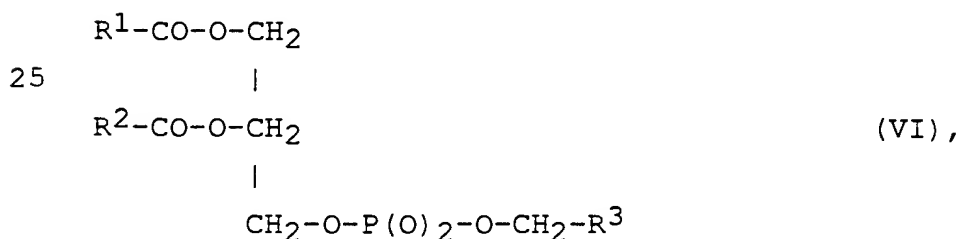
To the double bond of this maleimido group is in the next
 5 step the phospholipid covalently bound by a simple addition reaction to form the third layer.

Step 3: Formation of the third, proximal phospholipid layer

Phospholipids are applied for building up the covalently
 10 linked third, proximal lipid layer. A phospholipid comprises a glycerol bridge which links two long fatty acids (either saturated or unsaturated) with a polar head containing a phospho group. The fatty acids by convention occupy the 1st and 2nd position of the glycerol moiety while the phospho
 15 containing polar head group is in position 3. The phospho group is bound by an ester linkage to a lower alkyl group carrying a functional group able to covalently reacting with the functional group of the second layer. Such group is for example a group -CH₂-R₃ in formula VI.

20

Typical phospholipids forming the third layer and binding covalently to the second layer are for example of the formula



30 wherein R¹-CO and R²-CO independently from each other are rests of a fatty acid and R³ is a group CH₂-SH, CH₂-(O-CH₂CH₂)_n-SH, wherein n is from about 1 to 6, preferably 2, 3 or 4, CH₂NH₃⁺ or CH(COO⁻)NH₃⁺.

R¹-CO and R²-CO are rests of a natural or synthetic fatty acid having of from 12 to 20 carbon atoms, e. g. the rest of lauric, myristic, palmitic, stearic, arachidic, oleic, linolic, linolenic, or arachidonic acid. Such acids are found
5 in natural bilayer membranes.

Preferably compounds of the formula VI are selected from the group of naturally occurring or synthetically accessible phosphatidyl-thioethanols, phosphatidyl-ethanolamines and
10 phosphatidylserines, in particular e.g. DMPSH, DOPSH (see Abbreviations) or thiolipids of the class mentioned in WO 93/215280 and Lang et al., 1994, which citations are hereby incorporated by reference. Preferred are the phospholipids which form at ambient temperature the third layer in the
15 fluid state.

Reacting thiolipids with the maleimido groups of the second layer yields a proximal phospholipid layer covalently bound to the second linking layer through a thioether linkage.
20

Reacting phosphatidylethanolamines or phosphatidylserines with N-succinimidyl ester groups of the second layer results in a proximal phospholipid layer covalently bound to the second linking layer through amide linkage.
25

Reacting phosphatidylethanolamines or phosphatidylserines with carboxylic acid groups of the second layer in the presence of a coupling agent yields a proximal phospholipid layer covalently attached to the second linking layer through
30 an amide linkage. Water-soluble carbodiimides are preferably selected as coupling agents.

The reactions are carried out in a conventional manner. For forming the proximal phospholipid layer second functional
35 groups are conveniently contacted with a phase transfer

catalyst, e. g. a detergent solution of above mentioned phospholipids in aqueous media. The detergent OG (see Abbreviations) is preferably selected for this purpose. Due to its high critical micellar concentration (CMC) of 25 mM, OG can be easily removed from the resulting phospholipid layer by washing with aqueous media.

At this stage the proximal phospholipid layer is usually an imperfect layer as it covers only about 60 to 50% of the device surface. In the next step the imperfect layer is usually filled up and completed by incorporation of a surplus of phospholipids used in the next step.

Step 4: Formation of the fourth, distal lipid layer

The proximal phospholipid layers serves as a hydrophobic template for the non-covalent deposition of the fourth, distal lipid layer. Lipids of the distal lipid layer can be of natural or synthetic origin, but are preferably selected from the phosphatidylcholine group, preferably phosphatidylcholines with one or two unsaturated fatty acid chains, most preferably 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC).

The lipids of the distal lipid layer are deposited in a conventional manner, e. g. by the vesicle or mixed micelle fusion method, on the third layer to give a lipid bilayer structure. Vesicle fusion and mixed micelle fusion have been described by Lang et al. (1992), Lang et al. WO 93/215280, and Lang et al. (1994), respectively. These citations are hereby incorporated by reference.

Vesicle fusion comprises the formation of small unilamellar vesicles (liposomes) composed of the lipids which should form the distal lipid layer and applying them onto the proximal

phospholipid layer. This procedure results in the deposition of a lipid layer onto the proximal phospholipid layer. Excess vesicles are removed by washing, e.g. with buffer B.

5 Mixed micelle fusion comprises detergent dilution and the formation of an aqueous dispersion composed of the lipids envisioned to form the distal lipid layer and a detergent. OG is preferably selected for this purpose. The mixed micelle dispersion is desposited onto the proximal phospholipid layer
10 and is then several times (> 10) diluted in a 1:1 manner with an aqueous buffer. This procedure results in the deposition of a lipid layer onto the proximal phospholipid layer. For both procedures, care has to be taken that the resulting lipid bilayer is always covered with a layer of water.

15 About 40 to 50% of the device surface may remain uncovered in the previous step by the proximal phospholipid layer. However, this imperfect proximal phospholipid layer is completed and filled up with lipids upon deposition of the
20 distal lipid layer either by vesicle or mixed micelle fusion (detergent dilution). Consequently, the proximal lipid monolayer of the resulting lipid bilayer is composed of phospholipids covalently attached to the (second) linking layer, and non-covalently bound lipids of the same species
25 that constitute the distal lipid layer.

Step 5: Incorporation of receptor molecules into the lipid bilayer:

Lipids carrying chemically reactive groups may be part of the
30 distal lipid layer. Said functionalized lipids are incorporated into the distal lipid layer during lipid deposition. Hence, functionalized lipids are conveniently already constituents of the small unilamellar vesicles or mixed micelles applied for lipid deposition.

Lipids carrying functional groups include as headgroup functions for example maleimides, carboxylic acids, activated carboxylic acids, primary amines, and photoactivatable headgroups. Water-soluble biomolecules including enzymes, 5 antigens, antibodies, lectins, and oligonucleotides may be covalently immobilized on such functionalized distal lipid layers through formal chemical reactions including amide formation, formation of thioethers and insertion of photogenerated intermediates into chemical bonds of target 10 molecules.

Lipids bearing receptor molecules may be a constituent of the distal lipid layer. Such lipids are capable of non-covalently binding their respective water-soluble ligands to the distal 15 lipid layer. Such systems include biotinylated lipids for binding of avidin or streptavidin, avidin or streptavidin derivatives, lipid-bound peptides or proteins, antigens for the binding of their respective antibodies, glycolipids for the binding of their respective lectins, and cell receptor 20 ligands for the binding of their respective receptor proteins.

Lipids bearing carbohydrates (glycolipids) or polymeric headgroups may be part of the distal lipid layer. Said lipids 25 are known to reduce unspecific protein adsorption to lipid bilayer membranes. Glycolipids are selected from the group of phosphatidylinositols or from the group of gangliosides. Polymeric headgroups are preferably polyethylene glycols with molecular weights \leq 2000.

30 Membrane bound receptor molecules may be incorporated into the lipid bilayer. Said receptor molecules include proteins which are bound to the lipid membrane via a lipid anchor, or membrane proteins which cross the lipid layer once or several 35 times and thereby extend on either side of the lipid bilayer.

Transmembrane proteins are preferably incorporated into a lipid bilayer membrane which is decoupled from the device surface via a second linking layer carrying several hydrophilic oligooxyethylene spacer groups. This arrangement enables receptor proteins which extend beyond the membrane to adopt a configuration which is more closely conform to that found in nature and enables them to respond to the binding of a ligand in a correspondingly natural fashion. For incorporation into the lipid bilayer membrane, transmembrane receptor molecules are preferably inserted into micelles thus forming mixed micelles prior to application for forming the distal lipid layer by detergent dilution.

A solid device according to the invention comprises in particular such devices, wherein said (second) linking layer provides a distance from said surface to said lipid bilayer to allow for an aqueous layer between said surface and said lipid bilayer, wherein into the lipid bilayer are inserted biological receptor molecules, e.g. such which are selected from the group consisting of antigens, haptens, lectins, bioreceptors, such as neural receptor ligands, oligonucleotides and antibodies capable of biospecifically binding with their respective analyte.

The invention pertains in particular to a solid coated device, wherein the device is composed of waveguiding materials, such as mixtures of SiO_2 and TiO_2 , tantalum oxide (Ta_2O_5), hafniumoxide, zirconiumoxide, or gallium arsenide.

The invention pertains in particular to a solid device, wherein the device surface is composed of an electrically conductive material, such as a metal or metal oxide carrying OH groups.

The invention pertains in particular to a solid coated device, wherein the first layer is bound via a covalent silicium-oxygen bond to the surface of the device.

- 5 The invention pertains in particular to a solid coated device, wherein the second, linking layer is bound via a covalent carbon-carbon bond, or an ether, ester or amide bond to the first layer.
- 10 The invention pertains in particular to a solid device, wherein the third, proximal phospholipid layer is bound via a covalent thioether or amide bond to said second, linking layer.
- 15 The fourth, distal lipid layer is formed in particular from mixtures of bilayer forming lipids, glycolipids, and lipids with polymeric polar head groups, said lipids, after addition in the form of mixed micelles or liposomes to said third, proximate phospholipid layer, filling up and forming together
- 20 with said proximal phospholipid layer a bilayer, which may in particular contain reconstituted or surface-linked biologically active agents selected from the group consisting of antigens, haptens, antibodies, carbohydrates, extracellular proteins, trophic factors and bioreceptors including cell
- 25 receptor ligands, capable of biospecifically binding with cell surface constituents.

In another aspect the invention covers a process for the preparation of said solid coated device comprising the steps of

- 30 (1) covalently attaching a first layer carrying a first group of functional groups to the uncoated device,
(2) covalently attaching to the first functional groups of said first layer a second, linking layer carrying optionally protected second functional groups,

(3) if necessary, deprotecting said protected second functional groups, and covalently attaching a third, proximal phospholipid layer to said optionally deprotected second functional groups,

5 (4) non-covalently attaching a fourth, distal lipid layer to said third, proximal phospholipid layer, so that both lipid layers together form a bilayer, and

(5) optionally inserting into said lipid bilayer no-covalently bound receptor molecules.

10

All the steps are carried out in a conventional manner. The process covers in particular a step 5, wherein said fourth, distal lipid layer is formed by contacting said third, proximal phospholipid layer with mixed micelles or small
15 unilamellar vesicles. In another aspect the invention concerns the use of a present solid coated device as a biosensor.

The devices of this invention find particular application when used as biosensors with membrane incorporated receptor
20 molecules, which can selectively bind drugs, proteins, viruses etc. Water-soluble receptor molecules may be covalently linked to the lipid membrane, the lipid membrane acting in this particular arrangement predominantly as an interfacing layer, which suppresses unspecific analyte
25 binding (Lang et al., WO 93/215280).

Many naturally occurring receptor molecules are incorporated into cell membranes via a lipid-anchor or are intrinsic membrane proteins, whose polypeptide chains cross the lipid
30 layer of natural membranes once or several times. Examples of the first type are glycosyl-phosphatidylinositol anchored proteins like acetylcholinesterase and alkaline phosphatase. Members of the second type are channel forming proteins like nicotinic acetylcholine, GABA, glycine and 5-HT₃ (serotonin)
35 receptor, and G-coupled receptor proteins like the muscarinic

acetylcholine receptor and the beta-adrenergic receptor. The devices of this invention will find particular application in the reconstitution of membrane-associated receptor molecules into covalently attached supported lipid bilayers and their
5 exploitation as sensing elements in biosensors.

The devices of this invention find use in a variety of sensing devices, especially those wherein the lipid bilayer with incorporated receptor molecules is in intimate contact
10 with a transducer such that changes in electrical resistance and capacitance of an electrode upon which the bilayer is mounted can be monitored (Lang et al., WO 93/215280).

The devices of this invention find further use in sensing
15 devices wherein the lipid layer with incorporated receptor molecules is in intimate contact with a transducer such that the binding of ligand molecules to the receptors can be optically monitored. This is conveniently performed by measuring the changes in the effective refractive indices of
20 guided modes using waveguiding techniques.

The invention further concerns the use of a present device as implantation device for the human or animal body.

25 The ability to repel proteins and prevent cell growth on implant surfaces is a desired goal for many blood and tissue compatible applications. In this regard, covalently linked phospholipid bilayers on permanent (e.g. pace maker) or temporary implant devices (e.g. catheters) suppresses
30 undesired protein adsorption, protein deposition or cell adherence. The invention thus allows to covalently bind phospholipids on implant surfaces and to introduce phospholipid constituents into the second lipid layer which are effective in protein or cell repulsion.

A second beneficial application of the invention concerns lubrication between load bearing implant components. Friction forces in artificial metal-polymer joins which are currently in use, lead to material abrasion. As a consequence, abraded material accumulates at the implant/tissue interphase and
5 adverses healing. Binding of molecular glycolipid films onto metal-based artificial join surfaces allows to use metal-metal joins for load bearing implants. Covalently immobilized lipid bilayer films on either metal surface favorably
10 counteract abrasion through lubrication and retention of molecular solvent layers.

Many modifications and variations of the present invention are possible in light of the above teachings. It is therefore
15 to be understood that within the scope of the appended claims the invention may be practiced otherwise than as specifically described.

20 **Sources and Abbreviations used throughout the description and Examples:**

APTES	3-aminopropyl-triethoxysilane, Merck, purified by distillation under vacuum
biotin-DPPE	N-((6-(biotinoyl)amino)-hexanoyl)-1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphoethanolamine, Molecular Probes, Eugene, Oregon (USA)
25 buffer A	25 mM sodium phosphate buffer, pH 8.0
buffer Ab	66 mM sodium phosphate buffer, pH 7.0, with 100 mM NaCl added for antibody binding experiments
30 buffer B	25 mM sodium phosphate buffer, pH 6.8,
DMF	dimethylformamide

	DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine, Sigma
5	DMP SH	1,2-dimyristoyl-sn-glycero-3-phosphothioethanol, Avanti Polar Lipids, Alabaster/Alabama (USA)
	DOP SH	1,2-dioleoyl-sn-glycero-3-phosphothioethanol, Avanti Polar Lipids, Alabaster/Alabama (USA)
	DTNB	5,5'-dithio-bis-(2-nitro-benzenic acid)
10	IgG	immunoglobulin G
	IOS	integrated optical scanner
	lipo-peptide	N-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-(NANP) ₃ , G. Jung, Institut für organische Chemie, Universität Tübingen, Germany, (Metzger, Wiesmüller et al., 1991)
15		
	NANP	peptide sequence Asn-Ala-Asn-Pro
	OG	N-octyl-beta-D-glucopyranoside (Sigma or Bachem AG, Bubendorf / Switzerland)
20	POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids, Alabaster/Alabama (USA)
	SA	self-assembly
	SMP	N-succinimidyl 3-maleiminidopropionate [N-(3-maleimido)propionyloxy)-succinimid], Fluka
25		
	SPR	surface plasmon resonance

Further sources:

Tetradekanthiol Fluka in purum quality

- Monoclonal anti-(NANP)_n-antibody Sp3E9 raised against a
(NANP)₄₀ Dr. H. Matile, Hoffmann-La Roche AG,
Basel, Switzerland. The antibody was 95%
pure (SDS-PAGE)
- 5 Streptavidin Boehringer Mannheim AG, in BioChemika
quality
- C(NANP)_{6Y}, "(NANP)₆" synthesized by Dr. Anne Sévin, using
a solid phase strategy and Fmoc
protection (Atherton, Logan et al., 1979)
- 10 [³⁵S]-cysteine, specified activity range 20-150 mCi/mmol
Amersham
- water purified via an ion exchanger purification
train (Nanopure D4752 system,
Barnstead) with attached 0.2 mm filter
- 15 (Supor, DCF specification)

All other chemicals used were reagent grade.

- Waveguide instrumentation:** Planar optical waveguides incor-
porating an embossed grating with grating period $L = 1/2400$
20 mm were obtained from ASI AG, Zürich, Switzerland (type 2400,
ca.170 nm TiO₂:SiO₂ 2:1 waveguiding layer of refractive index
 n_F ca. 1.8 on AF 45 glass substrate of refractive index n_S
ca. 1.52). These sensor chips were measured with the
Integrated Optics Scanner IOS-1 from ASI. The conditions for
25 chemical surface modification were optimized using waveguides
made from the same material as the sensor chips but lacking
the grating coupler. A custom made open cuvette was used to
hold the reaction solution (200-400 µL) placed on top of the
grating coupler of the optical waveguide.

30

The following Examples serve as an illustration of the
invention, however, should not be construed as a limitation
thereof.

Example 1: Preparation of an optical biosensor containing a DOPS/POPC bilayer

Step 1: Covalent attachment of the first layer: Silanization of waveguide sensor chips with APTES

5 The above described waveguide sensor chips (ASI AG; 4.8 cm x 1.6 cm) are cleaned by incubating them for 5 min in a hot (90°C) 1:1:5 mixture of NH₄OH/H₂O₂/H₂O, followed by rinsing three times with double-distilled water. The chips are then treated for 5 min with a hot (90°C) 1:1:5 mixture of
10 HCl/H₂O₂/H₂O and again washed extensively with double-distilled water, then rinsed three times with acetone before being vacuum dried for 12 hours at ambient temperature.

Silanization is performed by incubating a clean and dry
15 single chip in 30 ml of dry toluene containing 0.5 ml (2.15 mmol) of APTES. After refluxing for about 3-4 hours the solvent is removed at the end of the reaction, and the chip is washed with chloroform (five times), acetone (twice), and methanol (five times). The silanized chip is dried under a
20 stream of nitrogen and kept in acetonitrile at 4°C until use. The number of 3-aminopropylsilanyl groups per sensor chip surface area is determined with ninhydrine as described by Sarin et al., 1981, and is about 1.6 nmol NH₂/cm² (100% covered).

25

Step 2: Covalent attachment of the second, linking layer: 3-Maleimidopropionylation of the amino group of the first layer
3-Aminopropyl-silanized sensor chips are removed from acetonitrile, dried under a stream of nitrogen and stored in
30 buffer A for at least 12 hours. The sensor chips are then placed within two tight fitting metal plates, the grating area on the waveguide surface being accessible by a circular teflon-lined 1.5 cm² opening in the cover metal plate. After washing twice with buffer A, the 3-aminopropylsilanyl groups

containing waveguide is treated with 200 μ l of a 25 mM solution of the heterobifunctional crosslinker SMP in buffer A/DMF 4:1 (v/v). After incubation for 30 min at ambient temperature, excess reagent is removed by washing once with
5 DMF and ten times with buffer B.

Step 3: Covalent attachment of the third, proximal lipid layer: Addition of the thiolipid DOPS to the maleimido double bond

10 DOPSH/OG mixed micelle solutions are prepared by dissolving a dried thiolipid DOPSH film (0.5 mg) in 500 μ l of a 50 mM solution of OG in buffer B. The presence of thiols in the dispersion is assayed by the development of yellow color after 1:1 mixing with Ellman reagent (10 mM DTNB in water,
15 Riddles et al., 1983).

Immediately after the modification with SMP the waveguide chip is assembled with the open O-ring cuvette and mounted on the turntable of the Integrated Optics Scanner (IOS-1). The
20 waveguide/cuvette assembly is rinsed twice with buffer B, then the baseline of the waveguide is recorded. The buffer is replaced by the mixed micelle solution and the thiolipid binding is monitored with the IOS-1. After different times of incubation (ranging from 30 minutes to 16 hours) at ambient
25 temperature, the waveguide surface is rinsed with buffer B. A surplus of the only physisorbed thiolipid DOPS is removed by two to three washings with each 200 μ l 50 mM OG. The thickness of the third layer is given in Table 1, No. 1.

30 **Step 4: Non-covalent attachment of the fourth, distal lipid layer: Formation of the DOPS/POPC bilayer**

The products of step 4 are treated a) with a vesicle dispersion (Lang et al., 1994), or b) with a mixed micelle

solution of POPC (Lang et al., 1994) to form a lipid bilayer by vesicle spreading.

a) The vesicle dispersion is produced by drying down a
5 chloroform solution of 1 mg of POPC under nitrogen, adding 50
µl of buffer B to the lipid film and sonicating the aqueous
mixture 3-4 times for 3 minutes in a bath-type sonicator
(Sonorex RK 102p) until a clear vesicle dispersion is
obtained. This dispersion is diluted with buffer B to a final
10 lipid concentration of 1 mg/ml. 200 µl of the vesicle
dispersion is placed on the grating region of the thiolipid-
containing waveguide chip of step 3 and the lipid adsorption
is measured with the IOS-1 until a stable layer is obtained.
Excess vesicles are removed without disturbing the formed
15 layer by diluting 1:1 (v:v) with buffer B 10 times, while
continuously maintaining the waveguide covered with buffer.

The thickness of the POPC layer as averaged over 9
experiments is shown in Table 1, No. 3. The average thickness
20 of the entire DOPS/POPC bilayer determined from 4 experiments
(like the one shown in Fig. 3) is given in Table 1, No. 9.

b) The mixed micelle solution is produced by dissolving 1 g
of POPC in chloroform, drying the solution under nitrogen
25 down to obtain a film of POPC which is dissolved in 1 ml of
50 mM of OG in buffer B to give a concentration of 1 mg/ml of
POPC. 200 µl of this solution is placed on the thiolipid-
modified waveguide of step 3. After 5 minutes, dilution is
started by adding 200 µl buffer B and removing 200 µl of the
30 sample. This procedure is repeated 10 times, allowing the
sample to equilibrate between the dilution cycles for at
least 1.5 minutes.

The average thickness of the POPC layer, determined from 3
35 experiments, is shown in Table 1, No. 4.

Example 2: Preparation of an optical biosensor containing a DMPS/POPC bilayer

In analogy to Example 1, Step 3, the thiolipid DMPS is added to the maleimido double bonds.

5

The results of 2 experiments are given in Table 1, No. 2.

In analogy to Example 1, Step 4, b) a DMPS/POPC bilayer is produced from the product of Step 3 and POPC.

10 The average thickness of 3 experiments of the POPC layer is shown in Table 1, No. 5 (from micelles).

Example 3: Preparation of an optical biosensor containing a DOPS/POPC/2% biotin bilayer (see Fig. 3)

15 In analogy to Example 1, Step 4, a), a DOPS/POPC/2% biotin bilayer is produced from the product of Step 3 and a mixture of POPC and biotin-DPPE.

20 The average thickness of the POPC/2% biotin layer of 6 experiments is shown in Table 1, No. 6 (from vesicles).

Example 4: Preparation of an optical biosensor containing a DMPS/POPC/2% lipopeptide bilayer (see Fig. 4)

25 The lipopeptide used is that shown under Sources and Abbreviations. In analogy to Example 1, Step 4, b), a DMPS/POPC/2% lipopeptide bilayer is produced from the product of Example 2, Step 3 and POPC/2% lipopeptide.

30 For experiments with lipopeptide, dilution of mixed micelle solutions is used exclusively for producing the second layer on top of the thiolipid layer. Mixtures of POPC and lipopeptide dissolved in chloroform: methanol 1:1 (v:v), are dried and subsequently dissolved in 50 mM of OG solution in buffer B (final concentration 1 mg lipid/ml solution). 200 µl

of this solution is placed on the thiolipid-modified waveguide of Example 3. After 5 minutes, dilution is started by adding 200 μ l of buffer B, mixing, and removing 200 μ l of the sample. This procedure is repeated 10 times, allowing the
5 sample to equilibrate between the dilution cycles for at least 1.5 minutes.

The average thickness of the POPC/2% lipopeptide layer determined from four experiments is shown in Table 1, No. 7 (from
10 micelles).

Example 5: Preparation of an optical biosensor containing DOPS/POPC/lipopeptide bilayer

In analogy to Example 3 a DOPS/POPC/lipopeptide bilayer is
15 produced from the product of Example 2, Step 3, and mixed micelles of POPC and different lipopeptide ratios varying from 0.5-4%.

The average thickness of the POPC/lipopeptide layer of 9
20 experiments is shown in Table 1, No. 8 (from micelles).

Example 6: Streptavidin binding to biotin-containing bilayers (see Fig. 3)

Non-covalent binding of Streptavidin to biotin-containing
25 bilayers of Example 3 is measured by injecting a solution of streptavidin in buffer B into the cuvette volume, to give final concentrations of 0.4 - 1.7 μ M streptavidin. Binding is allowed to take place for 5-45 minutes, then the unbound streptavidin is removed from the reaction solution by dilution
30 (10 times 1:1 with buffer B).

The results are shown in Fig. 3.

Example 7: Antibody binding to POPC membranes containing 2 mol% lipopeptide (see Fig. 4)

Antibody (monoclonal anti-(NANP)_n-antibody Sp3E9) binding to POPC bilayers of Example 4 containing 0-4 mol % of lipopeptide is initiated by injecting a solution of 0.3 mg/ml Anti-(NANP)_n antibody in Ab-buffer to give final antibody concentrations of 100-200 nM. The binding is allowed to continue for 15-40 minutes, then unbound antibodies are removed by washing with Ab-buffer.

Specifically bound antibodies are displaced from the membrane surface by adding 66 µl of a 0.6 mg/ml (NANP)₆ solution in Ab-buffer (final (NANP)₆ concentration 75 µM).

The results are shown in Fig. 4.

Example 8: Preparation of an implantation device containing a DOPS/POPC bilayer

Titanium implant surfaces, e.g. the headpiece or the corresponding pan of an hip joint, carrying a vapor deposited surface layer of titanium nitride are treated with 10 % nitric acid in distilled water during 20 minutes at 80 °C. This treatment generates hydroxyl functions on the surface of the titanium substrate. The surface is rinsed four times with bidistilled water.

Step 1: Covalent attachment of the first layer: Silanization of the implantaion device with APTES

Silanization of the activated surfaces is performed by incubating the surface in 150 ml of dry toluene containing 2.5 ml (10.75 mmol) of APTES. The solvent is removed at the end of the reaction, and the implant surface is washed with chloroform (five times), acetone, (twice), and methanol (five times). Headpiece and recipient pan surfaces are dried with

nitrogen and stored in acetonitrile at 4 °. Silanized device surfaces are washed twice with each 150 ml buffer A.

Step 2: Covalent attachment of the second layer: 3-Maleimidopropionylation of the amino group of the first layer:

The silanized device surface of Step 1 is treated with 200 ml of 25 mM heterobifunctional crosslinker SMP in buffer A/DMF 4:1 (v/v). After incubation for 30 min at ambient temperature, excess reagent is removed by washing once with DMF and ten times with buffer B.

Step 3: Covalent attachment of the third layer: Addition of a thiolipid to the maleimido double bond

Covalent thiolipid binding to the 3-maleimidopropyl group of the linker modified surfaces of Step 2 is carried out in situ using teflon coated counter part mimics to displace the solvent and thus reduce the total volume required for thiolipid binding. (Teflon coated pan mimics are used for headpiece modification. The Teflon coated counterparts are perforated for solvent (reagent) inlet. Inlets density: one perforation per cm², perforations are connected to a feeder tubing and a solvent delivery system. The setup allows homogeneous surface perfusion.) Thiolipid, e.g. DOPS or DMPS, and OG containing mixed micelle solutions are prepared by dissolving a dried thiolipid film (5 mg) in 5 ml 50 mM OG in buffer B. The reaction is carried out for 4 hours at ambient temperature and physically adsorbed lipid is removed by washing with 50 mM OG (twice) and buffer B (5 times).

Step 4: Non-covalent attachment of the fourth layer:

Formation of lipid/lipid bilayer

The second lipid layer is formed by incubation of the surface product of Step 3 with heterologous (soybean, egg

yolk), synthetic or autologous (extracted from red cells or fat tissue) lipids. Formation of the second lipid layer on top of the thiolipid layer is attained by dissolving the chosen lipid or lipid mixtures (e.g. egg PC) in
5 chloroform: methanol 1:1 (v:v). The lipid is dried and dissolved in 50 mM of OG in buffer B. Mounted in the counter part mimic, the lipid/OG solution is brought in contact with the thiolipid modified layer of Step 3 by the solvent delivery system. After 15 min, detergent dilution
10 is initiated by dispensing buffer B at a rate of 0.4 ml/min via the feeder tubings during 50 min. Bilayer covered joint surfaces are washed with and stored in saline until use.

15

20

25

Table 1: Characterization of lipid layers on modified waveguide surfaces by integrated optics

No	Thiolipid ^a	Phospholipid ^a (method)	Thickness [Å] ^b	number of experiments
Lipid layers on maleimide-modified waveguides				
1	<u>DOPSH</u>	----	13±5	8
2	<u>DMPSH</u>	----	11±1	2
3	DOPSH	<u>POPC</u> (vesicles)	28±7	9
4	DOPSH	<u>POPC</u> (micelles)	30±5	3
5	DMPSH	<u>POPC</u> (micelles)	31±5	3
6	DOPSH	<u>POPC/2% biotin</u> (vesicles)	36±4	6
7	DMPSH	<u>POPC/2% LP</u> (micelles)	27±5	4
8	DOPSH	<u>POPC/0.5-4% LP</u> (micelles)	31±5	9
9	<u>DOPSH</u>	<u>POPC</u> (vesicles)	47±8	4

^a Lipid bilayers were produced by first binding thiolipid from mixed micelle solutions to waveguides and subsequently assembling phospholipids or mixtures of phospholipids either with biotinylated lipids or with lipopeptides, by the method indicated in brackets. LP stands for (NANP)₃-lipopeptide.

^b Mean average thickness values given here refer to the lipid layers indicated in underlined letters, i.e. to incomplete mono- or complete bilayers. They were calculated using an index of refraction of $n_{Az} = 1.45$.

REFERENCES:

- 5 Bayerl, T.M. and Bloom, M. (1990), Physical properties of single phospholipid bilayers adsorbed to micro glass beads, Biophys. J. 58, 357-362.
- Chapman, D. (1993) Biomembranes and new hemocompatible materials. Langmuir 9, 39-45.
- 10 Contino, P.B., Hasselbacher, C.A., Ross, J.B.A., and Nemerson, Y. (1994) Use of an oriented transmembrane protein to probe the assembly of a supported phospholipid bilayer. Biophys. J. 67, 1113-1116.
- 15 Erdelen, C.H., Jullien, L., Ringsdorf, H., Merkel, R., and Sackmann, E. (1992) Tethered and mobile supported bilayers as model membranes. Poster presented on 4th European Conference on Organized Thin Films. Bangor, U.K.
- 20 Efimov et al., Phys. stat. sol. (a) 129, 483 (1992), Physico-chemical Properties of Plasma Deposited Silicon Nitride Films
- Gennis, R.B. (1989) Biomembranes. Molecular structure and function. Springer Verlag. N.Y.
- 25 Gmelin, Handbook of inorganic and organometallic chemistry, Silicon Suppl. Vol 5d1, Springer Verlag, Berlin, 8th edn. (1995)
- 30 Hong, H.-G., Jiang, M., Sligar, S.G., and Bohn P.W. (1994) Cysteine-specific surface tethering of genetically engineered cytochromes for the fabrication of metalloprotein nanostructures. Langmuir, 10, 153-158.

- Johnson, S.J., Bayerl, T.M., McDermott, D.C., Adam, G.W., Rennie, A.R., Thomas, R.K., and Sackmann, E. (1991), Structure of an adsorbed dimyristoyl-phosphatidylcholine bilayer measured with specular reflections of neutrons, 5 Biophys. J. 59, 289-294.
- Kalb, E., Frey, S., Tamm, L.K. (1992) Formation of supported planar bilayers by fusion of vesicles to supported phospholipid monolayers. Biochim. Biophys. Acta 1103, 307-316.
- 10 Kallury, K.M.R., Ghaemmaghami, V., Krull, U.J., and Thompson, M. (1989) Immobilization of phospholipids on silicon, platinum, indium/tin oxide and gold surfaces with characterization by X-ray photoelectron spectroscopy and time-of-flight 15 secondary ion mass spectroscopy. Anal. Chim. Acta 225, 369-389.
- Kiefer, H., Klee, B., John, E., Stierhof, Y.-D., and Jähnig, F. (1991), Biosensors based on membrane transport proteins, 20 Biosensors and Bioelectronics 6, 233-237.
- Kono, K., Yoshihiro, I., Kimuar, S., and Imanishi, Y. (1989) Platelet adhesion on to polyamide microcapsules coated with lipid bilayer membrane. Biomaterials 10, 455.
- 25 Lang, H., Duschel, C., and Vogel, H. (1994), A new class of thiolipids for the attachment of lipid bilayers on gold surfaces, Langmuir 10, 197-210.
- 30 Lang, H., Duschel, C., Grätzel, M., and Vogel, H. (1992), Selfassembly of thiolipid molecular layers on gold surfaces: optical and electrochemical characterization, Thin Solid Films 210/211, 818-821.

- McConnell, H.M., Watts, T.H., Weis, R.M., and Brian, A.A. (1986) Supported planar membranes in studies of cell-cell recognition in the innume system. *Biochim. Biophys. Acta* 864, 95-106.
- 5
- Metzger, J, Wiesmüller, K.-H., Schaudé, R., Bessler, W.G. and Jung, G. (1991), Synthesis of novel immunologically active tripalmitoyl-S-glycerylcysteinyl lipopeptides as useful intermediates for immunogen preparations, *Int. J. Pept.*
- 10 *Protein Res.* 37; 46-57.
- Riddles, P.W., Blakeley, R.L., and Zerner, B. (1983), Reassessment of Ellman's reagent, *Methods Enzymol.* 91, 49-60.
- 15 Roberts, G. (1990) *Langmuir-Blodgett Films*. Plenum Press. N.Y.
- Sarin, V.K., Kent, S.B.H., Tam, J.P., and Merrifield, R.B. (1981), Quantitative monitoring of solid-phase peptide
- 20 synthesis by the ninhydrin reaction
- Terrettaz, S., Stora, T., Duschl, C., and Vogel, H. (1993) Protein binding to supported lipid membranes: Investigation of the cholera toxin-ganglioside interaction by simultaneous
- 25 impedance spectroscopy and surface plasmon resonance, *Langmuir* 9, 1361-1369.
- Uzgiris, E.E. (1987) UV immobilized phospholipid bilayers, *Biochem. Biophys. Res. Comm.* 3, 1116-1121.
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What is claimed is:

1. A solid device carrying a coating which coating comprises
 - (a) a first covalently attached layer containing first
5 functional groups,
 - (b) to which first layer functional groups is covalently
attached a second, linking layer carrying second functional
groups,
 - (c) to which second, linking layer is covalently attached a
10 third, proximal phospholipid layer,
 - (d) to which third, proximal phospholipid layer is non-
covalently attached a fourth, distal lipid layer, so that the
proximal and the distal lipid layers together form a lipid
bilayer,
 - 15 (e) into which lipid bilayer are optionally inserted
receptor molecules.
2. A solid device according to claim 1, which is amenable for
electrical or optical signal detection and propagation.
20
3. A solid device according to claim 1, wherein said second,
linking layer provides a distance from said surface to said
lipid bilayer to allow for a water layer between said surface
and said lipid bilayer and for a bioreceptor molecule
25 extending to the inside of the lipid bilayer.
4. A solid device according to claim 1, wherein into the
lipid bilayer are inserted bioreceptor molecules.
- 30 5. A solid device according to claim 1, wherein said receptor
molecules are selected from the group consisting of antigens,
haptens, lectins, bioreceptors, such as cell receptor
ligands, oligonucleotides and antibodies capable of
biospecifically binding with their respective analyte.

6. A solid device according to claim 1, wherein the device surface is composed of glass, diamond or diamond-like materials, silicium, silicium dioxide (SiO_2), silicon
5 nitride, tantalum oxide (Ta_2O_5), titanium dioxide (TiO_2), titanium nitride, titanium carbide, platinum, tungsten, aluminum, or indium/tin oxide.
7. A solid device according to claim 1, wherein the device
10 surface is composed of waveguiding materials including mixtures of SiO_2 and TiO_2 , tantalum oxide (Ta_2O_5) hafnium, zirconium, or gallium arsenide.
8. A solid device according to claim 1, wherein the device
15 surface is composed of an electric current conductive material.
9. A solid device according to claim 1, wherein the device surface consists of a biocompatible material suited for
20 implantation into a human or animal, such as a biocompatible metal including titanium, aluminum, platinum, and platinum alloys, or a biocompatible organic polymer, including polyurethane, poly(methyl methacrylate), polyethyleneterephthalate (PET), or polytetrafluoroethylene (PTFE).
- 25 10. A solid device according to claim 1, wherein the first layer carries as said first functional groups hydroxyl, thiol, carboxyl or amino functional groups.
- 30 11. A solid device according to claim 1, wherein the second, linking layer carries as said second functional groups maleimides, carboxyl or activated carboxyl functional groups.

12. A solid device according to claim 1, wherein the third, proximal phospholipid layer is bound via a covalent thioether or amide linkage to said second, linking layer.

5 13. A solid device according to claim 1, for use as a biosensor.

14. A solid device according to claim 1, for use as a biocompatible implantation device.

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15. A solid biocompatible implantation device according to claim 14, comprising a biocompatible lipid bilayer.

16. A process for the preparation of a solid coated device
15 according to claim 1, comprising the steps of

(1) covalently attaching a first layer carrying a first group of functional groups to the uncoated device,

(2) covalently attaching to the first functional groups of said first layer a second, linking layer carrying optionally
20 protected second functional groups,

(3) if necessary, deprotecting said protected second functional groups, and covalently attaching a third, proximal phospholipid layer to said optionally deprotected second functional groups,

25 (4) non-covalently attaching a fourth, distal lipid layer to said third, proximal phospholipid layer, so that both lipid layers together form a bilayer, and

(5) optionally inserting into said lipid bilayer no-covalently bound receptor molecules.

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17. A process according to claim 16, wherein said fourth, distal lipid layer is formed by contacting said third, proximal phospholipid layer with mixed micelles or small unilamellar vesicles.

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18. A process according to claim 16, wherein the fourth, distal lipid layer is formed from mixtures of bilayer forming lipids, glycolipids, and lipids with polymeric polar head groups, said lipids, after addition in the form of mixed
5 micelles or liposomes to said third, proximal phospholipid layer, filling up and forming together with said third, proximal phospholipid layer a bilayer.

19. A process according to claim 16, wherein the said
10 micelles and vesicles contain reconstituted or surface-linked biologically active agents selected from the group consisting of antigens, haptens, antibodies, carbohydrates, extracellular proteins, trophic factors and bioreceptors including cell receptor ligands, capable of biospecifically binding
15 with cell surface constituents.

20. Use of a device according to claim 1 as biosensor.

21. Use of a device according to claim 1 as implantation
20 device for the human or animal body.

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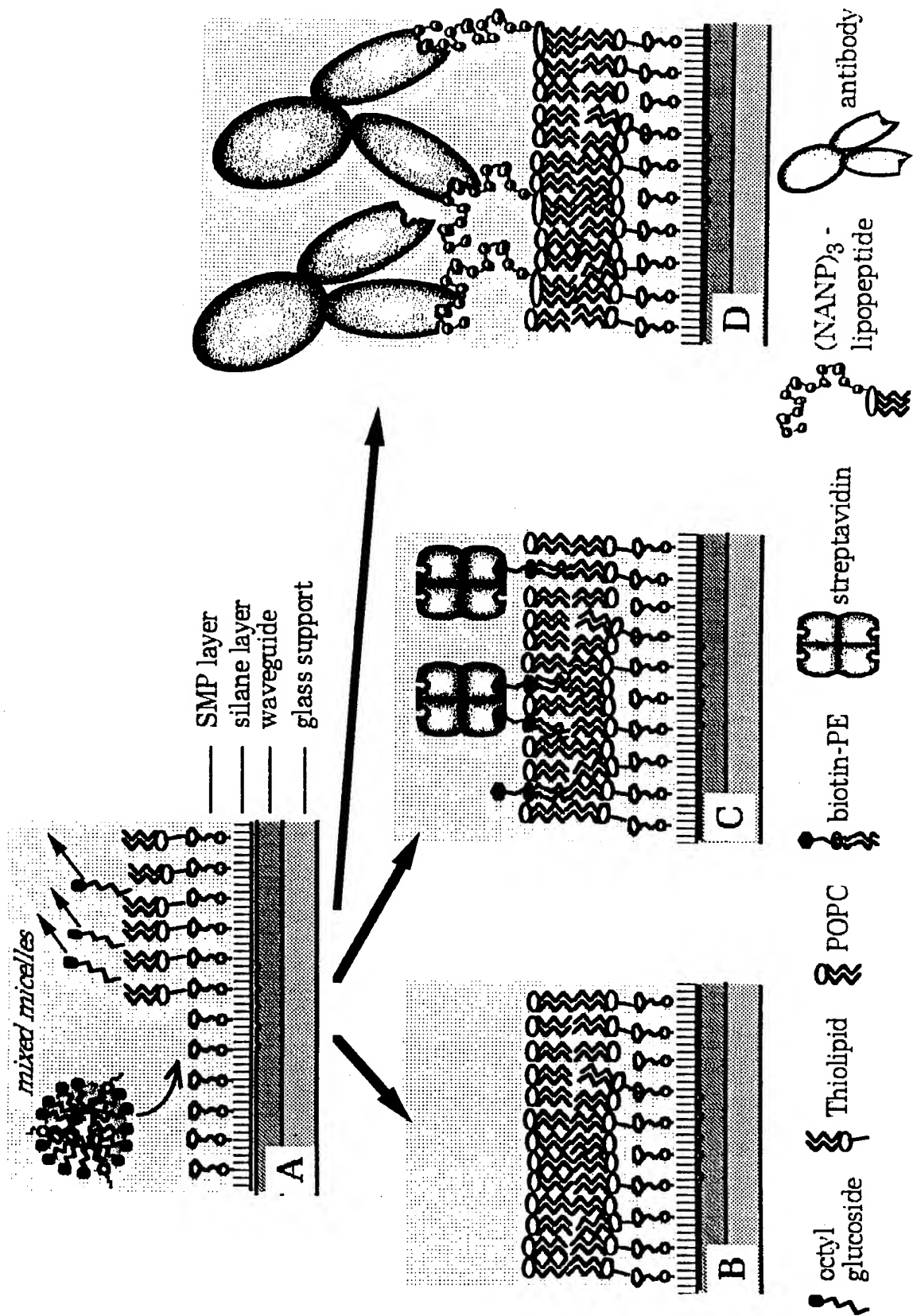


Fig. 1

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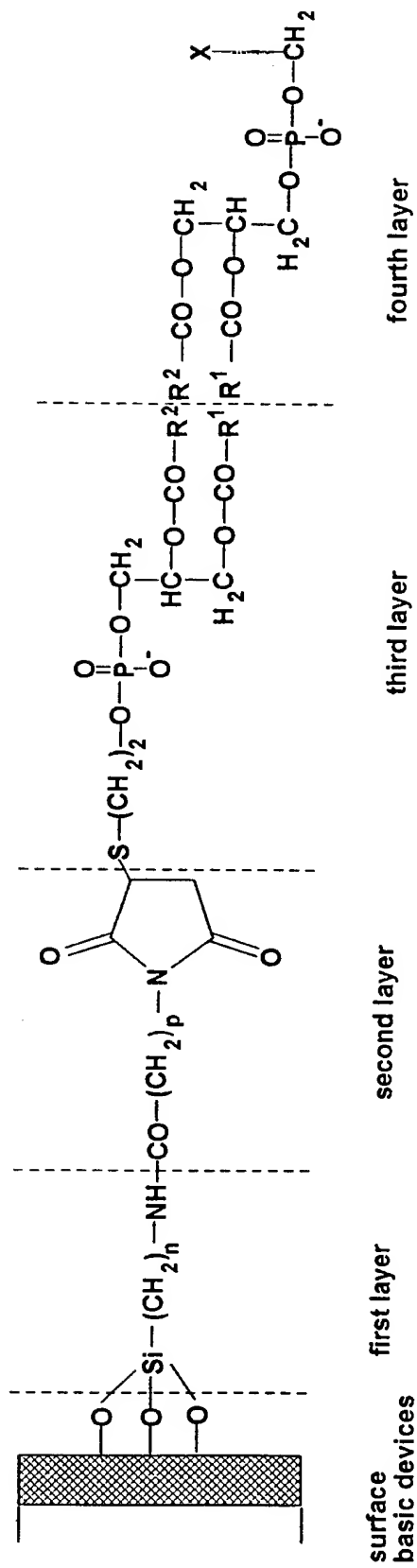


Fig. 2

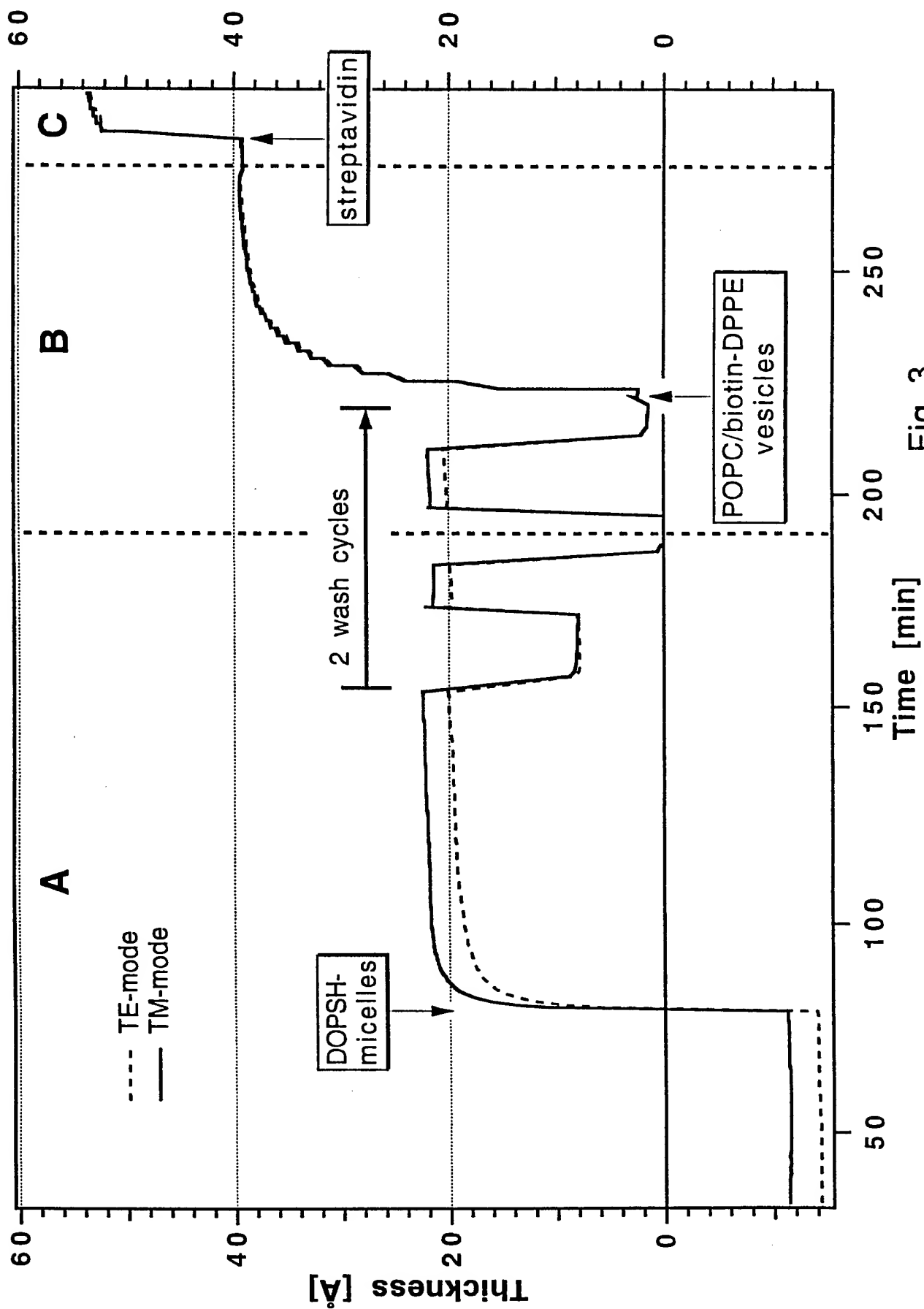


Fig. 3

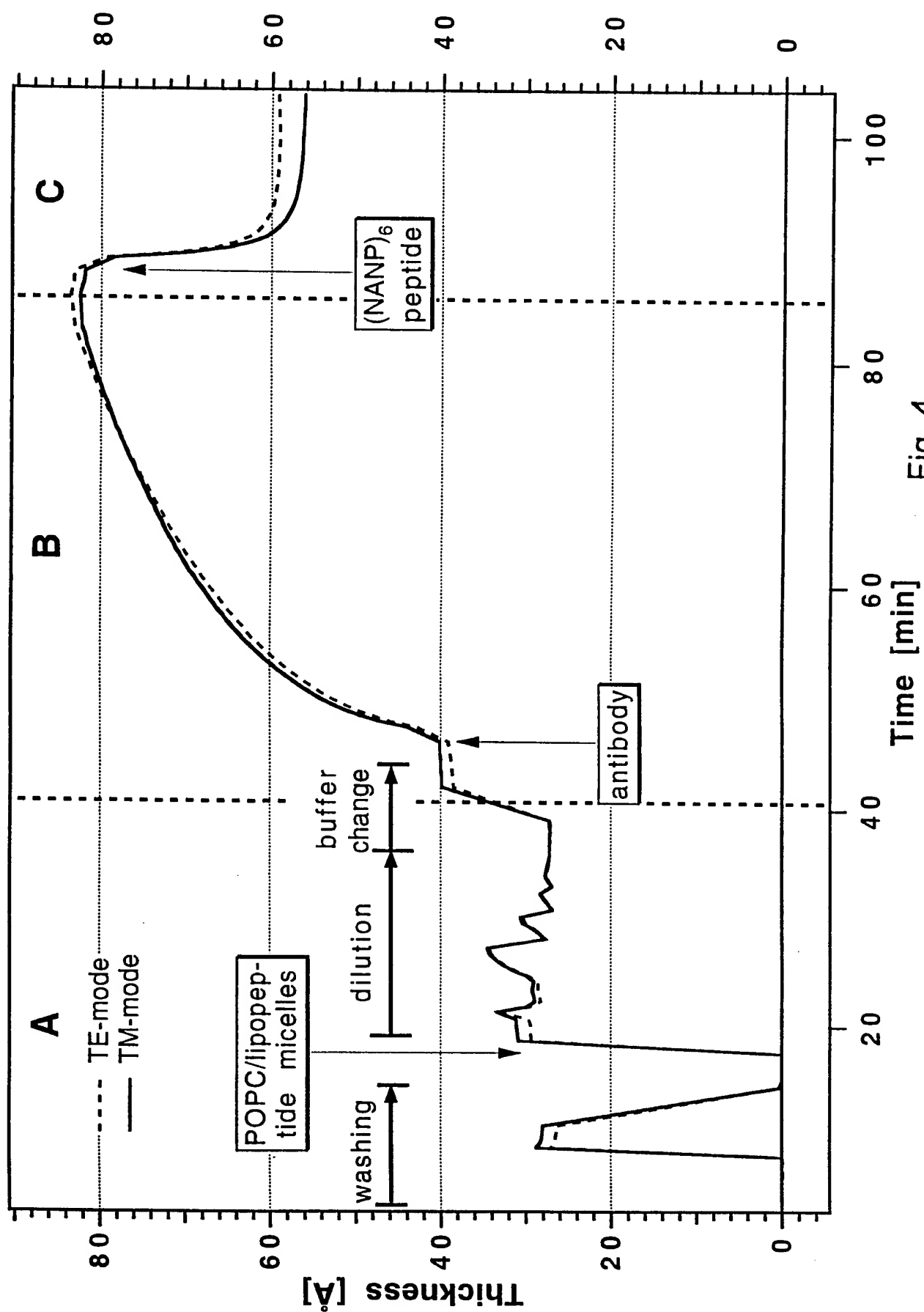


Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 96/00496

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 G01N33/543 C12Q1/00 G01N27/327 A61L27/00 A61L29/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N C12Q A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 441 120 (YEDA RES & DEV) 14 August 1991 see figure 2; examples 2,3 ---	1-21
X	WO,A,93 21528 (EUROP I OF TECHNOLOGY ; LANG HOLGER (DE); KOENIG BERND (CH); VOGEL) 28 October 1993 see figure 1; examples 6-8 ---	1-21
Y	WO,A,94 07593 (AUSTRALIAN MEMBRANE & BIOTECH ; UNIV SYDNEY (AU); RAGUSE BURKHARD ()) 14 April 1994 see examples ---	1-21
Y	CA,A,2 064 683 (CANADA MIN NAT DEFENCE) 27 September 1993 see figures; examples ---	1-21
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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

23 July 1996

Date of mailing of the international search report

20.08.96

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 96/00496

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,89 05977 (IGEN INC) 29 June 1989 see figures 11,12; example 6 ---	1-21
A	BIOSENSORS & BIOELECTRONICS, vol. 9, 1994, pages 337-341, XP002009043 M. REHAK ET AL: "Application of biotin-streptavidin technology in developing a xanthine biosensor based on a self-assembled phospholipid membrane." see the whole document ---	1,16,20, 21
A	LANGMUIR, vol. 10, no. 3, March 1994, pages 877-882, XP002009044 J. K. CULLISON ET AL: "A study of cytochrome c oxidase in lipid bilayer membranes on electrode surfaces." see the whole document -----	1,16,20, 21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 96/00496

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0441120	14-08-91	IL-A- 93020	29-06-95
		AT-T- 130938	15-12-95
		AU-B- 625017	25-06-92
		AU-B- 6924591	11-07-91
		CA-A- 2033776	10-07-91
		DE-D- 69114870	11-01-96
		ES-T- 2082867	01-04-96
		JP-A- 6090736	05-04-94
		US-A- 5204239	20-04-93

WO-A-9321528	28-10-93	EP-A- 0637384	08-02-95
		JP-T- 7508342	14-09-95

WO-A-9407593	14-04-94	AU-B- 5144493	26-04-94
		CA-A- 2145996	14-04-94
		EP-A- 0670751	13-09-95

CA-A-2064683	27-09-93	US-A- 5405766	11-04-95

WO-A-8905977	29-06-89	AU-B- 2949689	19-07-89
		AU-B- 669758	20-06-96
		AU-B- 5311094	10-03-94
		CA-A- 1308021	29-09-92
		EP-A- 0362304	11-04-90
		IL-A- 88751	24-06-94
